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TITLE: Chemotherapy necessitates increased immune control of HHVs: A cause of persistent inflammation enabling protracted fatigue in breast cancer survivors

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14. ABSTRACT The purpose of this work is to determine the incidence rate and relative risk in women who have undergone chemotherapy and have a high HHV load toward severe CTRF. We aim to determine whether immune cell burden induced by viral surveillance leads to severe fatigue in these retrospective and prospective cohorts. The progress of the past year of this award was dedicated to finding a university where the PI had a solid program and mentorship committee that supports the advancement of the PI and the research proposed here. Furthermore, as these data are collected over the next year, Medical University of South Carolina offers a dedicated and experienced team of breast cancer doctors and researchers who support the initial scope of this project and possess the capacity and resources to expand upon finding from these studies toward an ultimate goal of improved post-chemotherapy quality of life for breast cancer survivors.				
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INTRODUCTION:

This work hypothesizes that chemotherapy can permanently alter the balance between the immune system and chronic herpes virus infections and the resultant increase in inflammatory cytokines exacerbates CTRF. **Here we report our work in acquisition of and undergoing categorization of sera markers of inflammation coupled with viral infection status in long-term breast cancer survivors. We further discuss interests that have grown from this work and others regarding the balance of pro and anti-inflammatory mediators in breast cancer survivors and potential correlation with CTRF. Finally, we report the current and future directions of our prospective study of breast cancer patients undergoing chemotherapy and assays to determine HHV infections correlated with CTRF.**

Specific Aim 1: To determine whether the number of HHV infections and/or the type of HHV infection carried by an individual contributes to protracted fatigue in BC survivors.

Specific Aim 2: To monitor fatigue levels, HHV infections, and HHV-specific immunity in BC patients during chemotherapy to assess the impact of therapy on immune control of HHVs and CTRF outcomes.

BODY:

In the past year our progress on this project has been both significant and exciting. This research is focused on two cohorts of patients. Firstly, in our specific aim one we undertake viral and immunological parameter assays of a retrospective cohort of BC survivors and aim to correlate these parameters to patient reported CTRF. Secondly, in our specific aim two we are actively engaged in a prospective clinical study where samples are collected prior to, during, and post chemotherapy from BC patients. Our initial hypotheses focused on the role of HHVs, specifically CMV and EBV, and enhanced CTRF.

Specific Aim 1:

We are currently in the data collection and analysis phase of these data and we *strongly* aim to produce our first manuscript from these data for submission by December 2013. Samples arrived at MUSC in July 2013. Importantly, we initially expected our collaborator, Dr Kerri Winters, to send only baseline serum samples from breast cancer survivors that participated in three separate exercise intervention studies. Each study recorded patient reported fatigue scores at the time of serum collection (1, 2). Excitingly, Dr Winters was able to share with us her complete data set of samples from three separate exercise intervention studies, thus enabling us to investigate an extra parameter of viral sero-status, CTRF, and potential effect of exercise intervention. For these data we hypothesize that viral serum titer may increase or decrease, and/or IFN- γ and neopterin (markers of viral activity) will change accordingly post exercise intervention with a correlative change in CTRF score. This hypothesis is due to the enhanced anti-inflammatory atmosphere that is enabled by exercise interventions (3) as well as data that suggest CMV-specific T cells may activate in response to exercise (4).

Here we report our initial findings from analysis of n=27, n=20, n=20 subjects from study 1, study 2, and study 3, respectively. We have performed CMV, EBV, neopterin, and IFN- γ analysis on the analyzed samples listed in Table 1. We will assess VZV and HSV-1 sero-status on n=121 serum samples. We are currently trouble-shooting IP-10 flow-based assay for serum samples (for the time we have substituted IFN- γ as IP-10 is a secondary measure of IFN- γ production). Finally, at present we have a data agreement plan in preparation with Dr Winters to enable the release of coded patient fatigue data.

Preliminary data for CMV and virus specific immune parameters are presented here. We first measured sero-status to CMV from samples and categorized subjects as uninfected (-) or infected (+). Due to the additional post intervention time point we were similarly able to assess CMV sero-status post exercise intervention. (Table 1)

Table 1.	Study 1		Study 2		Study 3	
	CMV+	CMV-	CMV+	CMV-	CMV+	CMV-
Pre-Intervention	13	14	9	11	10	10
Post-Intervention	13	14	9	11	10	10
% CMV+	48%		45%		50%	
Samples Analyzed		27		20		20
Samples To Analyze		18		14		22

Our analysis of pre and post intervention of n=67 subjects showed that n=32 were CMV+ and n=35 were CMV-. Viral status did not change post exercise intervention (data points were collected 12 months after baseline data were collected). Therefore, no new CMV infections were introduced during the time of exercise intervention. Here we show data analysis for IFN- γ and neopterin for CMV+ versus CMV- subjects taken from baseline serum samples (Figure 1).

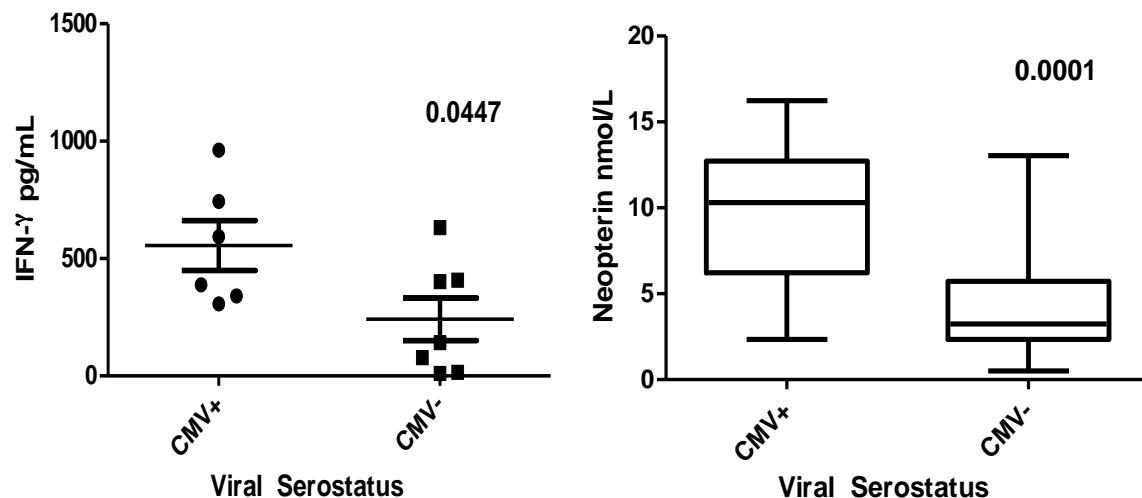


Figure 1. Subjects were grouped as CMV+ or CMV- and IFN- γ or neopterin levels were assayed. Data for sera IFN- γ and sera neopterin are significant between the two groups, p=.0447 and p=.0001, respectively.

These data demonstrate that CMV infection in latent form is associated with enhanced INF- γ and neopterin in serum. We will combine these data with our EBV, HSV-1, and VZV results to undertake analysis under advisement of the HCC Biostatistics Core to determine whether a correlation exists between HHV viral load and fatigue score as well as immune markers of viral activity and fatigue score.

Due to the availability of post-exercise intervention serum samples we are able to measure both viral titers prior to and post intervention. Given the accuracy of our IFN- γ and neopterin ELISA data we have analyzed pre and post IFN- γ and neopterin levels to correlate to viral status and fatigue score (data not shown). Preliminary analysis of CMV viral titers was performed for

analyzed serum samples (Table 1) pre and post three separate exercise interventions. We looked at percentage change from baseline of serum viral titers across the three interventions (Figure 2). Intervention 1 allowed for decreased CMV titer post intervention. Intervention 3 showed little to no change and intervention 2 showed an increase in CMV titers. First, it will be interesting to compare patient reported fatigue scores and viral status among the three interventions. Secondly, it is possible that exercise may increase active virus specific T cells and this may serve to enhance latent CMV activity (4).

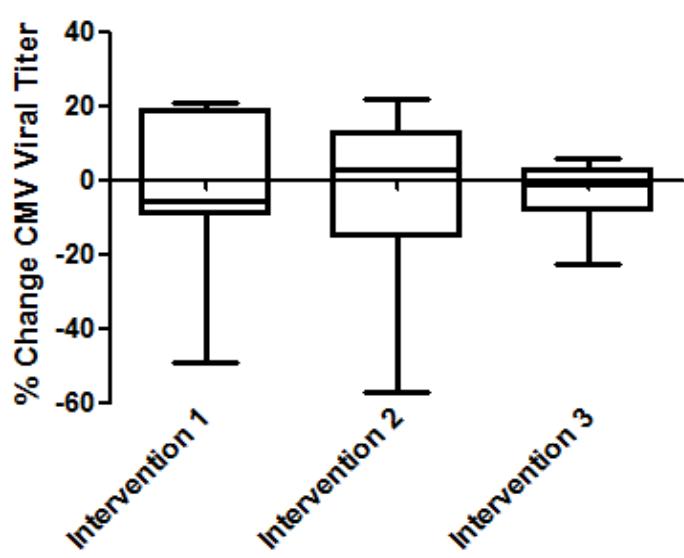


Figure 2. Percentage change in CMV-specific viral titers pre to post exercise intervention for 3 separate exercise interventions in breast cancer survivors.

We will continue our analysis of these data and will successfully complete SA1. Fatigue data will be employed in the coming weeks as our data agreement comes to fruition with Dr Winters and her team. Our biostatistics core is actively working with us to manage high-level statistical measures/models that will need to be implemented for multivariable (EBV, CMV, HSV-1, VZV, fatigue score, IFN- γ , neopterin) analysis.

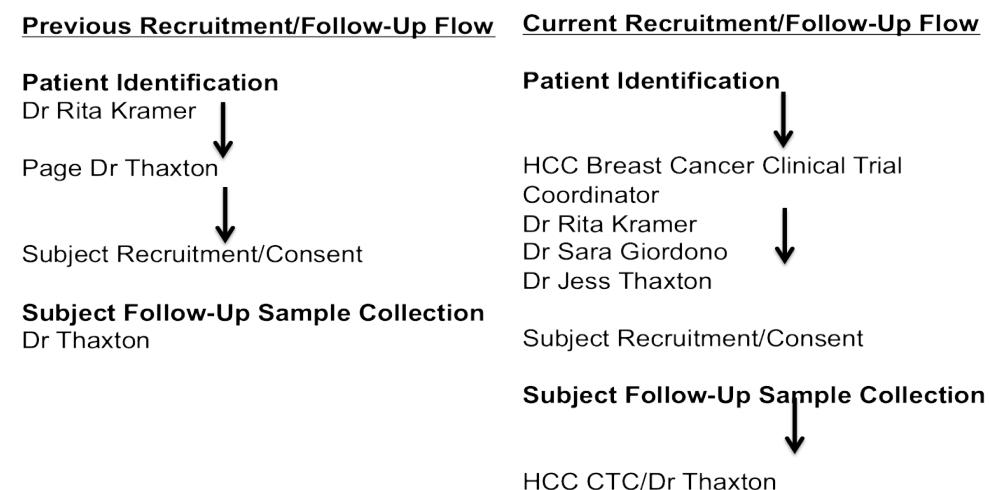
Specific Aim 2:

We aim to collect and analyze n=70 patient samples pre, during, and post-chemotherapy. Our IRB protocol was approved at the end of May 2013. At present we have recruited n=2 patients in 4 months for participation in this study. Consent, pre-chemotherapy viral survey, fatigue questionnaire, and blood were acquired and processed for each participant. Participants are followed through our online medical records system EPIC for return appointments and follow-up sample collection.

We are working aggressively to increase patient accrual for this study. During June –August our recruitment process was not satisfactory to us (“previous flow” described in Figure 3). We understand that clinical studies take time to find appropriate recruitment, consent, and acquisition/follow-up strategies. Enrollment has been particularly difficult because Dr Thaxton is a PhD with limited clinical access, thus dependent on breast oncologists for recruitment. Thus, we have established collaboration with the breast cancer clinical coordinator through the HCC clinical trials office. This relationship will allow better access between Dr Thaxton and clinical staff for patient recruitment. We are employing the following changes to increase and optimize our study flow:

- Dr Thaxton attends weekly Breast Cancer Tumor Board to increase awareness of this study among medical staff in HCC
- Assess the feasibility to open study recruitment to MUSC satellite facilities
- Enlistment of a second oncologist to recruit patients for this study, Dr Sara Giordono
- Enlistment of HCC Clinical Trials Office Director, Terri Matson to engage the HCC Clinical Trials Office to promote and help recruit subjects for our study
- Establishment of collaboration with new Clinical Trials Coordinator for Breast, Robin Bostick, to identify patients and recruit in concert with Dr Thaxton for this study
- Assessment of recruitment techniques: flyers, support groups, phone enrollment

Figure 3. Previous and Current Subject Recruitment/Follow-Up Strategy



KEY RESEARCH ACCOMPLISHMENTS

- Submission of prospective protocol to MUSC IRB
- Approval of prospective protocol by MUSC HCC Protocol Review Committee
- Approval of prospective protocol by MUSC IRB
- Approval of prospective protocol by HRPO
- Establishment of clinical coordination for patient recruitment with HCC CTO
- Submission of retrospective protocol to MUSC IRB
- Approval of retrospective protocol by MUSC IRB
- Approval of retrospective protocol by HRPO
- Recruitment of n=2 prospective patients, serum, PBMC isolation/storage
- Receipt of n = 121 serum samples from Dr Kerri Winters collaboration
- Completion of CMV sero-status assays for n=67 subjects, 2 time points
- Completion of EBV basal sero-status assays for n=67 subjects
- Completion of IFN- γ assays for n=67 subjects, 2 time points
- Completion of neopterin assays for n=67 subjects, 2 time points
- Establishment of breast cancer mentorship research team

Dr Stephen Ethier	Chair in Breast Cancer Diagnosis, Treatment, and Research
Dr Elizabeth Garret-Meyer	Director of Biostatistics, HCC
Dr Chanita Hughes-Halbert	Chair in Cancer Equity. Cancer Disparities
Dr Rita Munn Kramer	Breast Cancer Oncologist
Dr Zihai Li	Chair Microbiology & Immunology

REPORTABLE OUTCOMES:

- Attendance at IMPAKT 2013 Breast Cancer Conference
- Travel Award Recipient IMPAKT 2013 Breast Cancer Conference
- Abstract Presented: Jessica E Thaxton* Kerri Winters^{§#}, Ann Hill[§], Rita Kramer*, Zihai Li*
Chemotherapy necessitates increased immune control of HHVs: A cause of persistent inflammation enabling protracted fatigue in breast cancer survivors
- Manuscript in preparation:
Thaxton JE, Li Z, and K. Winters-Stone. *Correlation of HHV infections and cancer treatment related fatigue in breast cancer survivors*. 2013.
- Masters of Clinical Research Semesters FA12, SP13, SU13, FA13, cum: GPA 4.0

CONCLUSION:

We are directly in line with our SOW submitted in our October 2012 progress report (see below). Our hindrances for SAI have been overcome. In October 2012 we reported the potential pitfall regarding time in which it may take to obtain Dr Winters' serum samples for analysis for SAI. Samples for SA1 were sent to Massachusetts General Hospital for collaborator Kerri Winters' R21 research team to perform analysis. These samples were sent to MGH in April 2013 and were shipped to and received by Dr Thaxton in June 2013. Regardless of these facts, we have made significant progress in our study of these samples and our proposed analysis and do not foresee any further hindrances. We have a manuscript in preparation for this aim and are currently finishing analysis of HHVs (HSV-1 and VZV) and preparing a data agreement with Dr Winters for release of fatigue data to perform multivariable analysis for manuscript 1.

For SA II our subject recruitment has been lower than expected. We have described the steps we have implemented to increase subject recruitment for SAII (Figure 3) and continue to implement strategies to increase subject accrual.

Statement of Work:

September 2012-October 2012

1. Submit IRB to MUSC for expedited review to use samples from OHSU	Accomplished
2. Meet with MUSC research team to plan prospective patient sample collection	Accomplished
3. Prepare and submit full review IRB to MUSC for human subjects use approval	Accomplished

November 2012-February 2012

4. Receive retrospective cohort samples from OHSU	Accomplished
5. Perform HHV analysis on patient samples (VZV, EBV, CMV, HSV-1) to determine seropositive/negative status	Ongoing
6. IP-10 flow based assay, neopterin assay sample analysis	Ongoing

February 2013-July 2013

7. Coordinate coded fatigue data with serology and inflammatory protein results	Ongoing
8. Statistical consultation/analysis for fatigue score with HHV type or #	Ongoing
9. If necessary include total 285 subject data for enhanced significance	Accomplished
10. Preparation of data and production of manuscript 1	Ongoing
11. Patient recruitment and sample collection for SA2 begins	Accomplished

August 2013-January 2014

12. Assess patient recruitment rates and sample collection efficacy	Accomplished
13. Meet with study team for SA2 to revise and/or insure study maintenance	Accomplished
14. Patient recruitment and sample collection for SA2 continues	
15. HHV analysis of baseline samples for SA2 to determine sero-status	
16. PCR of CMV+/EVB+ for viral DNA in sero+ samples from 4 th cycle, 3-6 month follow up to determine if viral DNA is detectable	
17. IP-10 flow cytometry based assay	

February 2014-May 2014

18. Assess patient recruitment rates and sample collection efficacy
19. Meet with study team for SA2 to revise and/or insure study maintenance
20. Patient recruitment for SA2 is completed
21. HHV analysis of baseline samples for SA2 to determine sero-status
22. PCR of CMV+/EVB+ for viral DNA in sero+ samples from 4th cycle, 3-6 month follow
23. Coordinate patient fatigue data with HHV sero-status and inflammatory protein data
24. Statistical analysis/consultation for significance between SA1 parameters and SA2 (long-term vs short-term fatigue associations)
25. Preparation and submission of manuscript 2 from data (24)

June 2014-June2015

26. Sample collection and 1 year patient follow-ups complete
27. Finalize serum analysis of HHVs for all time points obtained (changes from baseline if detectable)
28. Finalize viral DNA PCR for EBV/CMV for all time points obtained (changes from baseline or 4th cycle to long-term follow-up time points if detectable)
29. Finalize IP-10, neopterin cytokine detection for all time-points obtained
30. Flow cytometry assays performed for immune cell changes between patient time-points collected for sero+ individuals: EVB/CMV peptide restimulation
31. Coordinate fatigue data with sero-status, viral-DNA outcomes, inflammatory cytokine status (IP-10, neopterin) and PBMC-virus specific immune activity data
32. Analysis/consultation for statistical significance for measured parameters (30)
33. Prepare, submit 2 manuscripts (manuscripts 3 and 4 from these data)

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